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Report

Reprogramming of Pancreatic β Cells into Induced Pluripotent Stem Cells

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Summary

Induced pluripotent stem (iPS) cells have been derived from fibroblast, stomach, and liver cultures at extremely low frequencies by ectopic expression of the transcription factors Oct4, Sox2, c-*myc*, and Klf4, a process coined direct or in vitro reprogramming [1–8]. iPS cells are molecularly and functionally highly similar to embryonic stem cells (ESCs), including their ability to contribute to all tissues as well as the germline in mice. The heterogeneity of the starting cell populations and the low efficiency of reprogramming suggested that a rare cell type, such as an adult stem cell, might be the cell of origin for iPS cells and that differentiated cells are refractory to reprogramming. Here, we used inducible lentiviruses [9] to express Oct4, Sox2, c-*myc*, and Klf4 in pancreatic β cells to assess whether a defined terminally differentiated cell type remains amenable to reprogramming. Genetically marked β cells gave rise to iPS cells that expressed pluripotency markers, formed teratomas, and contributed to cell types of all germ layers in chimeric animals. Our results provide genetic proof that terminally differentiated cells can be reprogrammed into pluripotent cells, suggesting that in vitro reprogramming is not restricted to certain cell types or differentiation stages.

Results and Discussion

Pancreatic β cells are mature, fully differentiated cells, whose defining characteristic is the expression of insulin. In vivo lineage-tracing studies have demonstrated that in healthy adult mice, the β cell population is maintained by self-duplication, not an adult stem cell [10]. Upon injury, insulin-producing β cells are also produced from facultative endocrine progenitors [11]. Importantly, these progenitors do not express insulin. Moreover, insulin-expressing β cells do not contribute to any other cell type in vivo [10]. Because of their easily defined identity and stable cell fate, pancreatic β cells are an ideal cell type with which to test whether iPS cells can be derived from a terminally differentiated cell type.

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We first tested whether β cells can be cultured under iPS cell culture conditions. To this end, we explanted pancreatic islets from 3- to 4-month-old mice that expressed GFP under the control of the *Pdx1* promoter [12]. Pdx1 expression in the postnatal pancreas is confined to β cells, in which it regulates insulin expression [13]. As shown in Figure 1, most islet cells were GFP^+ and maintained GFP expression in culture for at least 12 days. Rare GFP^- fibroblast-like cells appeared after ~1 week (Figures 1D and 1E). Most of these cells are probably derived from the pancreatic mesenchyme [12], whereas rare cells may also originate from β cells that have dedifferentiated in culture, as previously observed [14]. On the basis of the expansion of islets in culture, we estimated that β cells divided once before arresting. Incubation with a lentivirus constitutively expressing tdTomato showed that roughly 50% of GFP^+ islet cells (148 of 279 counted cells) became infected, compared with 80% of adult fibroblasts (209 of 261 cells), indicating that cultured islet cells can be transduced with lentivirus, albeit at a lower efficiency than fibroblasts (Figures 1F and 1G and data not shown).

To genetically mark β cells in the adult, we crossed RIP-Cre mice, in which the Cre gene is controlled by the β cell-specific rat *insulin* promoter [15], with ROSA26-lacZ reporter mice (Figure 2A). Immunostaining of pancreas sections showed that lacZ expression was restricted to $insulin^+$ cells contained within β islets, thus confirming the specificity of the transgene and excluding the possibility that non-β cells had been labeled (Figures 2B and 2C) [10]. On the basis of this observation, we conclude that most, if not all, cells with an active rat insulin promoter in the adult pancreas correspond to differentiated β cells.

Pancreatic islets from RIP-Cre/lacZ mice were isolated, explanted in culture for 2 days, and infected with doxycycline-inducible lentiviruses expressing Oct4, Sox2, c-*myc*, and Klf4 as well as a lentivirus constitutively expressing the reverse tetracycline-dependent transactivator (rtTA). Eighteen to twenty-four days after adding doxycycline to the cultures, colonies emerged that were visually different from pancreatic islets and resembled iPS colonies derived from fibroblasts (Figure 3B). The delayed kinetics with which iPS colonies appeared from pancreatic cultures compared with fibroblast cultures [9] may reflect the slow replication rate of islet cells. A total of 24 colonies were picked at day 24, and ~80% (19/24) of the colonies could be expanded in the absence of doxycycline, demonstrating independence of viral gene expression and activation of the endogenous pluripotency program, as has been seen previously [9, 16].

To verify the cellular origin of iPS cells, we stained the 19 expanded clones with the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (Xgal). Four of the clones (21%) stained homogenously positive with Xgal, demonstrating that they were derived from insulin-expressing β cells (Figure 2C), whereas all other clones were entirely $Xgal^-$. This number is significantly lower than the labeling efficiency of β cells with the RIP-Cre transgene (>80%), suggesting that $Xgal^-$ cells within explanted islets may be more easily reprogrammed or more efficiently infected by lentiviruses compared with β cells. Alternatively, the ROSA26 locus may have been silenced in some of the $Xgal^-$ iPS cell clones. iPS cells derived from β cells will be referred to as β iPSs.

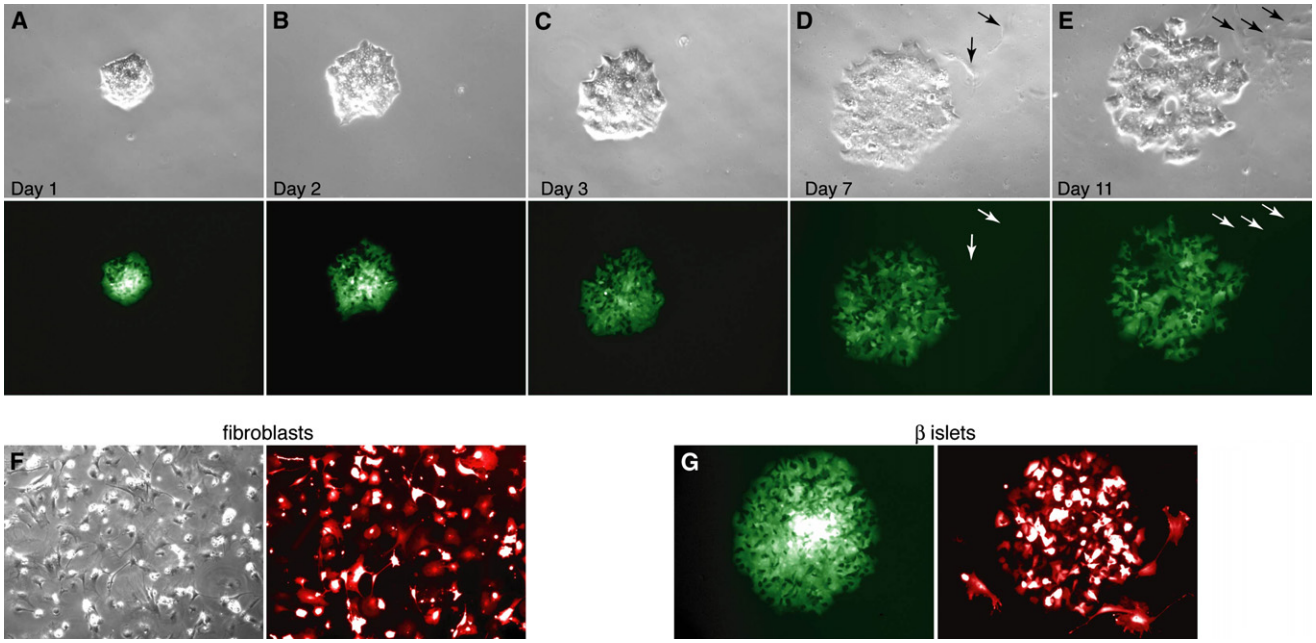

Figure 1. Culture and Viral Infection of Pancreatic Islets

(A–E) Bright-field (upper panel) and fluorescence (lower panel) images of a representative pancreatic islet isolated from Pdx1-GFP mice and imaged after the indicated culture periods. Note that most cells in the islet remain GFP$^+$ and that islet cells stop expanding after one to two cell divisions. Rare GFP$^-$ fibroblast-like cells present in the cultures are indicated by arrows in (D) and (E).

(F and G) Images of tail-tip fibroblasts (F) and islet cells from Pdx1-GFP mice (G) infected with a lentivirus constitutively expressing the red-fluorescent protein tdTomato.

We estimated the reprogramming efficiency to range between 0.1% and 0.2% (for calculation, see Experimental Procedures), which is similar to that seen in fibroblast cultures with the same lentiviral system [9]. To rule out the possibility that the RIP-Cre transgene becomes activated during the reprogramming process, we generated iPS cells from tail fibroblasts of RIP-Cre/lacZ mice in which the *insulin* promoter is silenced. Out of more than 50 iPS clones analyzed, none stained positive for Xgal, thus confirming the tightness of the RIP-Cre/lacZ system (data not shown).

All four β iPS clones expressed the pluripotency markers SSEA1, Nanog, Sox2, and Oct4 at the protein level (Figures 3D–3F and data not shown), indicating faithful molecular reprogramming. Real-time quantitative PCR analysis showed that β iPSs expressed endogenous c-*myc*, *Klf4*, *Oct4*, and *Sox2* at levels comparable to ESCs, whereas viral gene products were essentially undetectable (Figure S1 available online).

To test the differentiation potential of β iPS cells, we injected the four cell lines into the flanks of SCID mice. All β iPS lines formed teratomas, which on histological examination showed

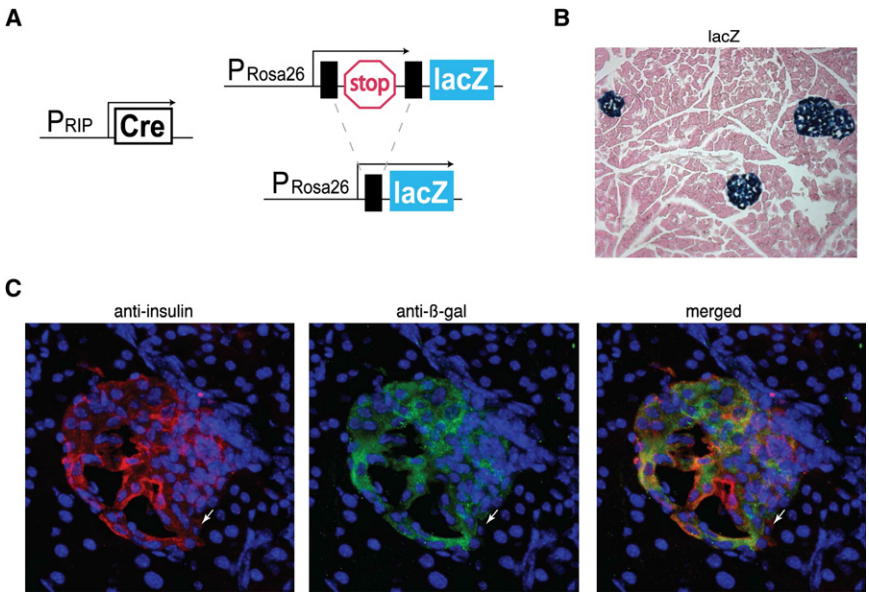

Figure 2. Characterization of RIP-Cre/lacZ Pancreas

(A) Scheme illustrating β cell-specific activation of lacZ expression in RIP-Cre/lacZ mice.

(B) Frozen pancreas sections from a RIP-Cre/lacZ mouse after Xgal staining shows islet specific labeling.

(C) Immunofluorescence staining of a RIP-Cre/lacZ islet section with antibodies against insulin (red) and β-galactosidase (green) demonstrates exclusive expression of β-galactosidase in insulin-positive β cells. The position of a rare β cell not expressing β-galactosidase is highlighted by white arrows.

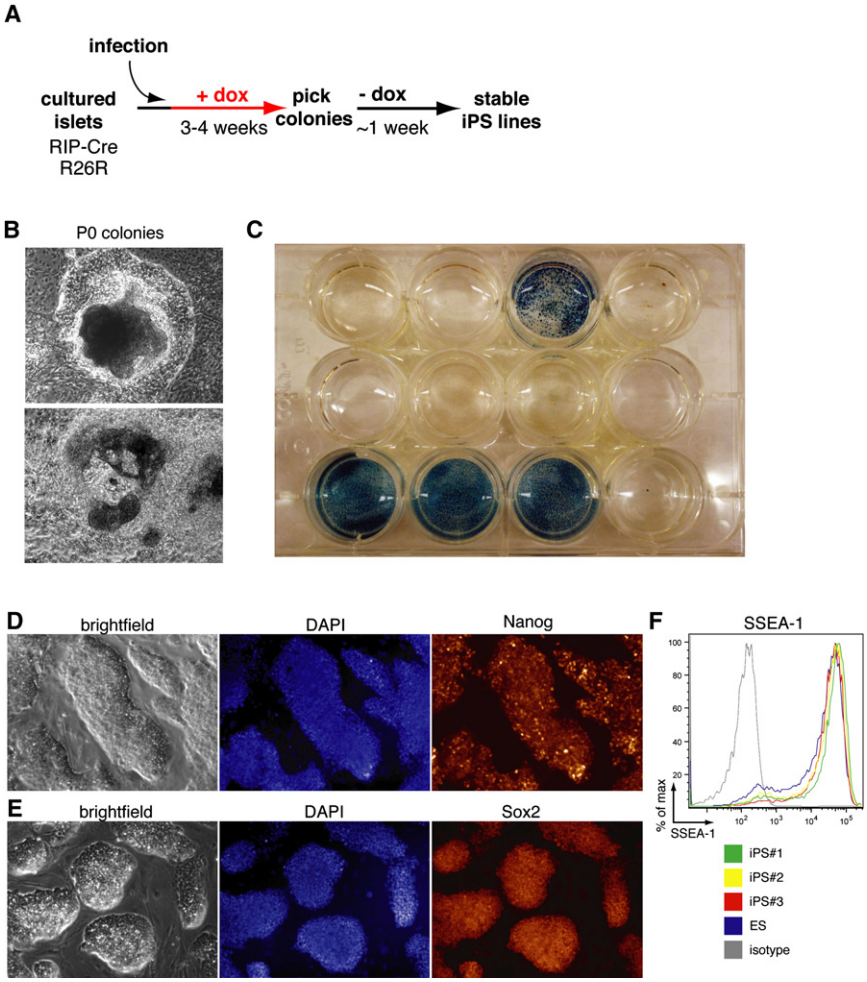

Figure 3. Generation of β iPS Cells

(A) Experimental outline.

(B) Bright-field images showing two typical iPS colonies obtained after lentiviral infection of islet cultures.

(C) Xgal staining of iPS lines derived from islets shows that 4 of the 12 depicted lines express β-galactosidase from the ROSA26 promoter.

(D and E) Bright-field and fluorescent images of β iPS cells stained for the pluripotency markers Nanog (D) and Sox2 (E). Nuclei were counterstained with DAPI.

(F) FACS histograms of three β iPS cell lines and an ESC line stained for SSEA-1 (colored lines) or with an isotype control (gray line).

differentiated cell types representative of all three germ layers, including glandular structures, muscle fibers, keratinized epithelia, and cartilage (Figures 4A–4E). Next, we injected three of the four iPS clones into blastocysts to assess their potential to contribute to normal development. All clones gave rise to E14.5 fetal chimeras and neonatal animals that were highly chimeric on the basis of whole-mount staining for lacZ activity, and cross sections of individual organs showed widespread tissue contribution (Figures 4F–4J and data not shown). One surviving chimera showed obvious coat-color chimerism but was cannibalized by its littermates at three weeks of age (data not shown). Together, these analyses demonstrate that the β iPS cells were reprogrammed to a pluripotent state.

Our findings allow three major conclusions. First, using genetic-lineage analysis, we demonstrate that cells from a defined cellular lineage can be reprogrammed into iPS cells that broadly contribute to tissues in chimeras. Second, our data show that reprogramming is not only confined to mesodermal derivatives, which have been used previously, but is also possible with an endodermally derived cell type. This is in agreement with a recent report showing reprogramming of stomach and liver cells into iPS cells [1]. Third, terminal differentiation, at least in the β cell lineage, does not restrict a cell's potential to be reprogrammed by the four reprogramming factors c-*myc*, Klf4, Oct4, and Sox2. In a previous report, iPS cells have been derived from genetically marked, albumin-expressing liver cells [1]. Because albumin is also expressed in hepatic progenitor and stem cells [17–19], this experiment did not unequivocally demonstrate the reprogramming of differentiated cells.

Our data indicate that the consistently low efficiency of iPS cell derivation from somatic cells is unlikely to reflect the reprogramming of rare stem or progenitor cells present in the starting cell population, suggesting that reprogramming is a universal process that is not restricted to certain cell types or differentiation stages. This does not exclude the possibility, however, that adult stem and progenitor cells are more amenable to in vitro reprogramming than differentiated cells. In agreement with this, pro B cells have been reported to be more easily reprogrammed into iPS cells than mature B cells, which required additional genetic manipulation of the B-cell-specific transcription factor Pax5 [20]. We believe that stochastic epigenetic-remodeling events are necessary for successful reprogramming, which results in the low overall reprogramming efficiencies. The requirement for such remodeling events or the frequency at which they occur might be cell type specific. Thus, it is conceivable that the genetic or pharmacologic manipulation of epigenetic modifiers may significantly increase reprogramming efficiencies in different cellular contexts.

Experimental Procedures

Mice

Derivation and handling of Pdx1-GFP, RIP-Cre, and R26R-lacZ mice were described previously. We crossed RIP-Cre mice with R26R-lacZ mice to obtain RIP-Cre/lacZ animals. Genotyping was done by PCR with the following oligonucleotides for RIP-Cre: 5′-TAGCACCAGGCAAGTGTTTG-3′ and 5′-ATGTTTAGCTGGCCCAAATG-3′. Genotyping for ROSA26 was done as described [21].

Isolation of Pancreatic Islets

The pancreas was perfused through the bile duct with digestion solution (low-glucose DMEM [GIBCO] with 10 mM HEPES [GIBCO], 0.25 mg/ml Liberase RI [Roche], and 0.1 mg/ml ovalbumin trypsin inhibitor [Roche]), dissected, and incubated at 37°C for 20 min. Cold washing solution (low-glucose DMEM with 10 mM HEPES, 10% FBS [Hyclone], and 0.1 mg/ml OTI) was added, and islets were centrifuged, washed twice, and filtered through a 500 μm diameter wire mesh. Islets were centrifuged, washed twice in washing solution, resuspended in Histopaque 1077 (Sigma), and vortexed. The islet suspension was carefully overlaid with washing solution

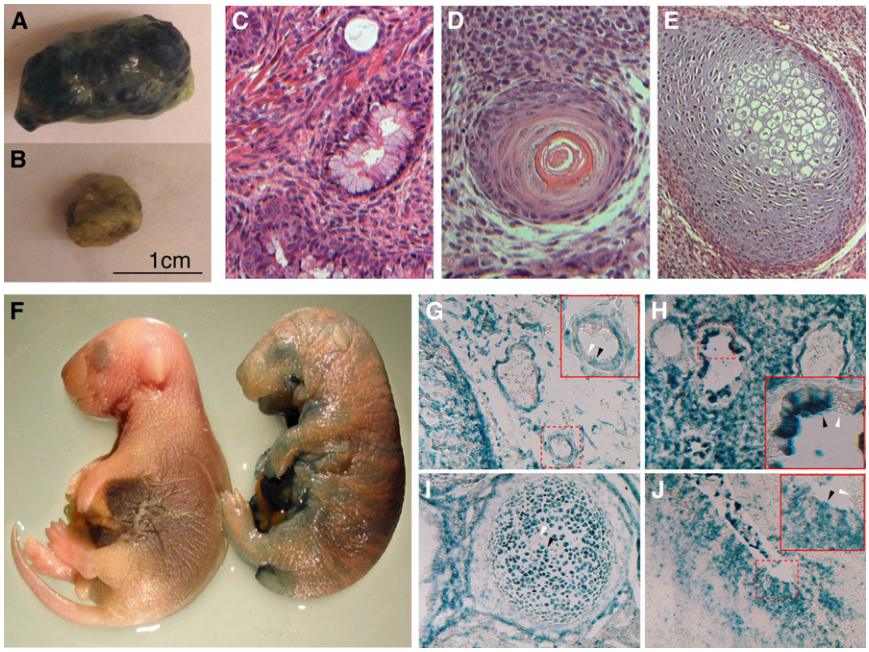

Figure 4. Developmental Potential of β iPS Cells

(A and B) Teratomas derived from β iPS (top image) as well as from fibroblast-derived iPS cells without a lacZ transgene (bottom image) were used for whole-mount Xgal staining. The light-blue staining seen in the control teratoma is due to weak background β-galactosidase activity.

(C–E) Histological sections of teratomas derived from β iPS cells subjected to H&E staining (without Xgal staining) show muscle and glandular cells (C) and keratinized epithelium (D) as well as cartilagous tissue.

(F) Images of newborn wild-type (left) and chimeric mice derived after blastocyst injection of β iPS. The tail of the chimeric mouse was used for establishing fibroblast cultures.

(G–J) Images of Xgal-stained sections from a β iPS-derived newborn chimera show both $Xgal^+$ (iPS-derived, black arrowheads) and $Xgal^-$ (blastocyst-derived, white arrowheads) cells in the vasculature (G), the bronchiolar epithelium of the lung (H), the cartilage of the rib (I), and neuronal tissue in the brain (J). Insets (red frames) show close-ups of chimeric areas.

(without serum) and centrifuged for 20 min at 10°C, separating islets from exocrine tissue. The islet layer was collected at the interface, pelleted, and washed twice. Finally, pure islets were handpicked under a dissecting scope, pelleted, washed, and cultured in ES medium on laminin coated plates.

Calculation of β Cell Reprogramming Efficiency

The number of islets that attached 1 day after seeding was determined to be ~500 islets per 35 mm plate. On the basis of an average number of 100 β cells per islet (K.B. and D. Melton, unpublished data), and a labeling efficiency of 80% in RIP-Cre mice [15], we calculated the total number of $Xgal^+$ cells present in the cultures to be ~40,000. Because ~50% of cells were infected by individual lentiviruses (Figure 1), we calculated the number of $Xgal^+$ β cells infected by all four viruses to be 2500 ($40{,}000 \times 0.5^4$). Out of 2500 cells, we obtained four $Xgal^+$ iPS clones at an efficiency of 0.16%.

Teratoma Formation

iPS cells were harvested by trypsinization, preplated onto untreated culture plates for removal of feeders as well as differentiating cells, and injected into the flanks of NOD/SCID mice, with ~5 million cells per injection. Mice were sacrificed 3 weeks later, and teratomas were isolated and processed for histological analysis.

Production of Chimeric Mice

Female BDF1 mice were superovulated with PMS and hCG and mated to BDF1 stud males. Zygotes were isolated from females with a vaginal plug 24 hr after hCG injection. After 3 days of in vitro culture in KSOM media, blastocysts were identified, injected with iPS cells, and transferred into pseudopregnant recipient females. Pups were delivered by Cesarean section at day 19.5 and nurtured by foster mothers.

Xgal Stainings

Cultured cells were fixed with 0.2% glutaraldehyde for 15 min and incubated in X-gal staining solution (0.1 M phosphate buffer with 2 mM $MgCl_2$, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide containing 1 mg/ml X-gal [5-bromo-4-chloro-3-indolyl-β-D-galactosidase]) for 12–16 hr at 37°C. Teratomas and newborn mice were fixed overnight in 1.5% paraformaldehyde (PFA) and washed with PBS before incubation in X-gal staining solution. For analysis of tissue chimerism, PFA-fixed newborn mice were equilibrated in 30% sucrose solution and frozen in OCT compound. Ten micrometer cryosections were prepared and incubated in X-gal staining solution overnight.

Immunofluorescence

iPS cells were cultured on pretreated coverslips, fixed with 4% PFA, and permeabilized with 0.5% Triton X-100. The cells were then stained with primary antibodies against mOct4 (Santa Cruz, sc-8628), mSox2 (Chemicon, AB5603), and mNanog (Abcam, ab21603); this was followed by staining with the respective secondary antibodies conjugated to Alexa Fluor 546 (Invitrogen). Nuclei were counterstained with DAPI (Invitrogen). Cells were imaged with a Leica DMI4000B inverted fluorescence microscope equipped with a Leica DFC350FX camera. Images were processed and analyzed with Adobe Photoshop software.

Flow Cytometry

Cells were harvested and incubated with either an antibody against SSEA-1 (MC-480, Developmental Hybridoma Bank) or an IgM isotype control, subsequently incubated with APC-conjugated mouse anti-mouse IgM antibody (II/41, eBiosciences), and analyzed on LSR2 (BD Biosciences). Dead cells were excluded by staining with DAPI. Data were analyzed with FlowJo software (Tree Star).

Viral Production and Cell Transduction

The generation and structure of tet-inducible lentiviruses expressing c-*myc*, Klf4, Sox2, and Oct4 has been described in detail elsewhere [9]. In brief, a tet-inducible lentivirus termed LV-tetO was generated by replacement of the ubiquitin promoter elements from the FUΔGW vector [22] with tetO sequences. cDNAs for c-MYC (T58A mutant), Klf4, Oct4, and Sox2 were isolated from pMIG or pMX vectors and cloned into LV-tetO. The lentivirus constitutively expressing tdTomato was a kind gift of Dr. Niels Geijsen, MGH. To produce infectious viral particles, we transfected 293T cells cultured on 10 cm dishes with the LV-tetO vectors (11 μg) together with the packaging plasmids VSV-G (5.5 μg) and Δ8.9 (8.25 μg) using Fugene (ROCHE) transfection reagent. Viral supernatants were harvested on 3 consecutive days starting 24 hr after transfection, yielding a total of ~30 ml of supernatant per virus. Viral supernatant was concentrated ~100-fold by ultracentrifugation at 20,000 rpm for 1.5 hr at 4°C and resuspension in 300 μl PBS. Viral concentrates were stored at −80°C. Infections were carried out in 1 ml ES medium containing 5 μg/ml polybrene with 5–10 μl of each viral concentrate per 35 mm plate. Fibroblasts were infected at passages 1 or 2 at a density of ~200,000 cells/plate. Primary islets cells were infected 2 days after seeding them at a density of ~500 islets/plate. The medium was replaced 24 hr after infection and supplemented with 1 μg/ml doxycycline another 24–48 hr later. Irradiated feeders cells were added to the pancreatic cultures ~10 hr after viral induction.

RNA Isolation and qPCR

RNA was isolated from cells with the TriPure reagent (Roche), and this was followed by RNA clean up with the RNeasy Minikit (QIAGEN). cDNA was produced with the First Strand cDNA Synthesis Kit (Roche). Real-time quantitative PCR reactions were set up in triplicates with the Brilliant II SYBR Green

QPCR Master Mix (Stratagene) and run on an Mx3000P QPCR System (Stratagene). Primer sequences are listed in Table S1.

Supplemental Data

One figure and one table are available at http://www.current-biology.com/cgi/content/full/18/12/890/DC1/.

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